

MEASURING PAVLOVIAN APPETITIVE CONDITIONING IN HUMANS
WITH THE POSTAURICULAR REFLEX

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Abstract

Despite its evolutionary and clinical significance, appetitive conditioning has been rarely investigated in humans. It has been proposed that this discrepancy might stem from the difficulty in finding suitable appetitive stimuli that elicit strong physiological responses. However, this might also be due to a possible lack of sensitivity of the psychophysiological measures commonly used to index human appetitive conditioning. Here, we investigated whether the postauricular reflex – a vestigial muscle microreflex, which is potentiated by pleasant stimuli relative to neutral and unpleasant stimuli – may provide a valid psychophysiological indicator of appetitive conditioning in humans. To this end, we used a delay differential appetitive conditioning procedure, in which a neutral stimulus was contingently paired with a pleasant odor (CS+), while another neutral stimulus was not associated with any odor (CS-). We measured the postauricular reflex, the startle eyeblink reflex, and skin conductance response (SCR) as learning indices. Taken together, our results indicate that the postauricular reflex was potentiated in response to the CS+ compared with the CS-, whereas this potentiation extinguished when the pleasant odor was no longer delivered. In contrast, we found no evidence for startle eyeblink reflex attenuation in response to the CS+ relative to the CS-, and no effect of appetitive conditioning was observed on SCR. These findings suggest that the postauricular reflex is a sensitive measure of human appetitive conditioning and constitutes a valuable tool for further shedding light on the basic mechanisms underlying emotional learning in humans.

Descriptors: Appetitive conditioning; Postauricular reflex; Eyeblink reflex; Startle; Reward; Odors

1. Introduction

Learning to predict the presence of potentially harmful or beneficial events in the environment is a critical adaptive function that enables organisms to shape appropriate behaviors fostering survival and reproduction. This kind of learning principally occurs through Pavlovian aversive and appetitive conditioning processes. In Pavlovian conditioning, the organism learns to associate an environmental stimulus (the conditioned stimulus, CS) with a motivationally salient aversive or appetitive stimulus (the unconditioned stimulus, US) through one or several contingent pairings (Pavlov, 1927; Rescorla, 1988).

While aversive conditioning has been extensively studied both in animals and humans (e.g., Delgado, Olsson, & Phelps, 2006; LaBar & Cabeza, 2006; Phelps & LeDoux, 2005), appetitive conditioning has been rarely investigated systematically in humans (Andreatta & Pauli, 2015; Hermann, Ziegler, Birnbauer, & Flor, 2000; Martin-Soelch, Linthicum, & Ernst, 2007). This paucity and asymmetry is rather surprising given that Pavlovian appetitive processes are considered to play a central role in reward processing (Berridge & Robinson, 2003; Pool, Sennwald, Delplanque, Brosch, & Sander, 2016) and to represent a crucial mechanism in the etiology, maintenance, and treatment of several major psychiatric conditions, including depression, addiction-related, and eating disorders (Martin-Soelch et al., 2007). It has been proposed that this discrepancy might be explained by the difficulty in finding appropriate appetitive stimuli that are able to elicit physiological responses that are similarly intense to the ones elicited by the aversive USs (e.g., electric stimulations) used in aversive conditioning (Hermann et al., 2000; Martin-Soelch et al., 2007), thereby resulting in potentially subtler effects (see Rescorla & Wagner, 1972). However, this discrepancy might also stem from a possible lack of sensitivity of the psychophysiological measures commonly used to systematically detect physiological changes induced by appetitive conditioning.

In line with this suggestion, human appetitive conditioning has generally been successfully evidenced using subjective measures (e.g., US expectancy and CS valence ratings; Van Gucht, Baeyens, Vansteenwegen, Hermans, & Beckers, 2010; Van Gucht, Vansteenwegen, Van den Bergh, & Beckers, 2008), behavioral measures (e.g., reaction times; Pool, Brosch, Delplanque, & Sander, 2014; Pool, Delplanque, et al., 2014; Van Gucht et al., 2008), or brain activity (e.g., Delgado, 2007; Franken, Huijding, Nijs, & van Strien, 2011; Gottfried, O'Doherty, & Dolan, 2002, 2003; Klucken et al., 2009; Prévost, McNamee, Jessup, Bossaerts, & O'Doherty, 2013), whereas the use of peripheral physiology measures (e.g., skin conductance response, SCR) has mainly yielded mixed or inconclusive results (see, e.g., Hermann et al., 2000). Developing psychophysiological indicators of appetitive conditioning thus constitutes an important purpose to eventually remedy the scarcity of knowledge about key mechanisms involved in emotional learning in humans.

In this vein, Andreatta and Pauli's (2015) study recently suggested that the startle reflex – an automatic defensive response to a sudden, intense, and unexpected stimulus – might be a putative index of human appetitive conditioning. In this study, the authors implemented a concurrent differential aversive and appetitive conditioning paradigm, in which three types of CS were used: One stimulus (aversive CS+) was associated with an electric stimulation (i.e., aversive US), one stimulus (appetitive CS+) was paired with sweet or salty food (i.e., appetitive US), and another stimulus (CS-) was not associated with any US. Overall, the aversive CS+ was rated as more negative and more arousing than the CS-, and elicited enhanced SCRs, while the appetitive CS+ was rated as more positive and also induced larger SCRs than the CS-, but was not rated as more arousing. Of particular interest, the startle eyeblink reflex was potentiated in response to the aversive CS+ compared with the CS-, whereas it was attenuated in response to the appetitive CS+, thereby replicating key findings obtained in rodents (e.g., Koch, Schmid, & Schnitzler, 1996). These results concurred with

prior research in the human startle literature indicating that the startle eyeblink reflex is specifically potentiated in response to unpleasant stimuli and attenuated in response to pleasant stimuli (Lang, Bradley, & Cuthbert, 1990). It has been however argued that the startle eyeblink response is primarily an index of the defensive motivational system, being hence optimal for studying aversive processes, but is not ideally suited for indexing appetitive processing (Dichter, Benning, Holtzclaw, & Bodfish, 2010). Although it is widely accepted that the startle eyeblink reflex does index defensive responding, mixed findings have been indeed reported regarding its role as an indicator of appetitive responding (Dillon & LaBar, 2005; Jackson, Malmstadt, Larson, & Davidson, 2000; for a review, see Grillon & Baas, 2003). Therefore, it remains unclear to what extent the startle eyeblink reflex is the most appropriate measure of appetitive conditioning in humans: The attenuation of this reflex may reflect an inhibition of defensive responding rather than appetitive responding *per se*.

In contrast, the postauricular reflex (PAR) has previously been suggested to provide a reliable index of appetitive processing (Benning, Patrick, & Lang, 2004; Sandt, Sloan, & Johnson, 2009). The PAR is a vestigial muscle microreflex in humans that serves to pull the ear backward and upward (Bérzin & Fortinguerra, 1993; Gray, 1901/1995). As for the eyeblink reflex, the PAR can be elicited with an acoustic startle probe. However, the PAR latency is faster than the eyeblink reflex latency (9-11 ms vs. 45-50 ms, respectively; Hackley, Woldorff, & Hillyard, 1987), suggesting that these two reflexes do not share the same underlying neural circuitry (Hackley, 2015). Importantly, a key aspect of the PAR lies in its sensitivity to affective modulation. Accumulating evidence has demonstrated that the PAR magnitude is potentiated during presentation of pleasant stimuli relative to neutral or unpleasant stimuli (Aaron & Benning, 2016; Benning, 2011; Benning et al., 2004; Dichter et al., 2010; Gable & Harmon-Jones, 2009; Hackley, Muñoz, Hebert, Valle-Inclán, & Vila, 2009; Hebert, Valle-Inclán, & Hackley, 2015; Hess, Sabourin, & Kleck, 2007; Johnson,

Valle-Inclán, Geary, & Hackley, 2012; Sandt et al., 2009), and in particular during viewing of appetitive images, such as food or erotic scenes (Sandt et al., 2009). These observations support the view that the PAR is an index of appetitive processing and accordingly suggest that the PAR may constitute a suitable psychophysiological measure for indexing human appetitive conditioning.

The current study therefore aimed to test whether appetitive conditioning may be measured with the PAR in humans. To this end, we applied a differential appetitive conditioning procedure, in which two initially neutral stimuli were presented. During the initial habituation phase, the two stimuli were presented without being reinforced. In the subsequent acquisition phase, one stimulus (CS+) was systematically paired with a pleasant odor (US), while the other stimulus (CS-) was not associated with any odor. We used a pleasant odor as US because pleasant odors have been shown to be an efficient primary reinforcer to trigger appetitive conditioning in humans (Gottfried et al., 2002, 2003; Pool, Brosch, et al., 2014; Pool, Brosch, Delplanque, & Sander, 2015). During the final extinction phase, the US was no longer delivered. The PAR, the startle eyeblink reflex, and SCRs were measured concurrently during all the conditioning phases as putative psychophysiological indices of appetitive conditioning, thus enabling a systematic comparison thereof. Subjective ratings were additionally collected after the conditioning procedure to assess learning at the subjective level. Our main hypothesis was that the PAR magnitude would be potentiated in response to the CS+ compared with the CS- during acquisition. Based on previous findings (Andreatta & Pauli, 2015), we also expected the CS+, in comparison with the CS-, to elicit larger SCRs, and a startle eyeblink reflex attenuation during acquisition.

2. Method

2.1 Participants

Sixty-three volunteers participated in the study, which was approved by the Faculty of Psychology and Educational Sciences Ethics committee at the University of Geneva. They received either partial course credit or monetary compensation for their participation. The sample size was determined prior to data collection with the aim of recruiting approximately 60 participants and based on previous research investigating the PAR in humans (Gable & Harmon-Jones, 2009; Hebert et al., 2015; Sandt et al., 2009). Eight participants were excluded from the analyses due to technical problems. The final sample consisted of 55 participants (34 women and 21 men), aged between 18 and 40 years old (mean age = 25.27 ± 5.56 years). From this sample, four participants (3 women and 1 man) were further excluded from the SCR analysis because of technical problems with the SCR recordings.

2.2 Stimuli and apparatus

Conditioned stimuli (CSs). The CSs were two neutral geometric figures commonly used in human conditioning paradigms (Gottfried et al., 2002, 2003; Pool, Brosch, et al., 2014; Pool et al., 2015; see Figure 1A). Each geometric figure served either as the CS+ or as the CS-, this assignment being counterbalanced across participants.

Unconditioned stimulus (US). The US consisted of a pleasant odor selected among a set of 17 different odors (Firmenich SA, Geneva, Switzerland; see Table 1). The odor that the participant rated as the most pleasant and intense was selected as the US for the appetitive conditioning procedure. More precisely, the most pleasant odor was chosen if its intensity was evaluated above or equal to a predefined threshold (i.e., 50 on a scale going from 0 to 100). In case the most pleasant odor's intensity was rated below this threshold, the second most pleasant odor was selected if (1) its intensity was rated as higher than the most pleasant odor's and (2) the pleasantness difference score between the most pleasant and second most pleasant odor was below or equal to 10. Otherwise, the most pleasant odor was chosen. Given the high and inherent variability of affective responses to odors across individuals (e.g., Ferdenzi et al.,

2013), this procedure was warranted to ensure that the selected odor was pleasant, sufficiently intense, and had rewarding properties for the participant, thus constituting an appropriate appetitive US. During both the US selection and appetitive conditioning procedures, the odors were released through a custom-made, computer-controlled olfactometer with an airflow fixed at 1 L/min delivering the olfactory stimulation rapidly, without thermal and tactile confounds, via a nasal cannula (see Ischer et al., 2014; Pool, Brosch, et al., 2014; Pool et al., 2015; Pool, Delplanque, et al., 2014).

Acoustic startle probe. The acoustic startle probe was a 50-ms white noise burst (105 dB) with a nearly instantaneous rise time (< 1 ms). The startle probe was presented binaurally through loudspeakers and delivered between 5 and 6 s after CS onset, or between 6 and 7.5 s after CS offset during intertrial intervals (ITIs).

2.3 Procedure

Prior to coming to the laboratory, participants were requested to refrain from eating before the experiment, which took place between 8.30 am and 12.30 pm. This procedure aimed to increase the likelihood that participants were in a hunger state, thereby optimizing the chances of the olfactory US to be rewarding, as is typically done in animal (e.g., Koch et al., 1996) and human (Andreatta & Pauli, 2015) appetitive conditioning studies.

Upon arrival at the laboratory, participants read and signed an informed consent form. They were then invited to provide background information, such as their age and gender, and to indicate their hunger level on a Likert scale from 1 (*not hungry at all*) to 10 (*very hungry*). Participants reported a mean hunger level of 5.75 ($SD = 2.44$). The skin conductance electrodes and the nasal cannula were next attached to them. Subsequently, participants performed the US selection procedure, in which the various odors (see Table 1), along with odorless air, were delivered to them in a randomized order. Each trial started with a 3-s countdown followed by an inspiration cue that indicated to participants to breathe in evenly.

The odors were released 0.5 s before the inspiration cue for a duration of 1.5 s. Participants were then asked to rate each odor according to its subjective pleasantness and intensity on visual analog scales (VASs) going from 0 (*extremely unpleasant* on the pleasantness VAS or *not perceived* on the intensity VAS) to 100 (*extremely pleasant* on the pleasantness VAS or *extremely strong* on the intensity VAS). Each trial ended with an ITI whose duration was adapted as a function of participants' rating pace (i.e., the ITI duration lasted for 15 s minus the time the participant took to rate the odor, with a minimal duration of 0.5 s).

INSERT TABLE 1 ABOUT HERE

Once the US selection procedure was completed, the electrodes for measuring the PAR and the startle eyeblink reflex were placed on participants. The room light was also turned dim to facilitate the acoustic startle reflex (Grillon, Pellowski, Merikangas, & Davis, 1997). Before the start of conditioning, 10 acoustic startle probes were delivered with an interstimulus interval randomly varying between 10 and 20 s to reduce the initial startle reactivity. The differential appetitive conditioning paradigm used a delay conditioning procedure and was composed of three contiguous phases (see Figure 1B). The habituation phase comprised four unreinforced presentations of each one of the two CSs. During the acquisition phase, each CS was presented nine times. Each CS+ trial co-terminated with the pleasant olfactory US, which was released 6.5 s after CS+ onset for a duration of 1.5 s (see Figure 1C), while the CS- trials were paired with odorless air. The extinction phase consisted of nine presentations of each CS, and no olfactory US was delivered during this phase. During all the conditioning phases, the CSs were presented for 8 s with an ITI ranging from 12 to 15 s, during which a fixation cross was presented onscreen (see Figure 1C). An inspiration cue indicating to participants to breathe in evenly was presented on each trial 7 s after CS onset (see Figure 1C). Startle probes were delivered on an equal number of trials for each CS (2 out of 4 during habituation, 6 out of 9 during acquisition, and 6 out of 9 during extinction).

Additional startle probes were presented during ITIs (2 during habituation, 6 during acquisition, and 6 during extinction) between 6 and 7.5 s post-CS offset in order to decrease their predictability (see Figure 1C).

INSERT FIGURE 1 ABOUT HERE

After the extinction phase, participants completed CS-US contingency and CS liking ratings to assess their awareness of the reinforcement contingencies and the evaluative effects of appetitive conditioning, respectively. In this procedure, the CSs were presented again to participants and were accompanied by a VAS. For CS-US contingency, participants were asked to rate to what extent the stimulus was predictive of the pleasant odor delivery on a VAS going from 0 (*never*) to 100 (*always*). For CS liking, participants were asked to rate to what extent the stimulus was unpleasant or pleasant on a VAS going from 0 (*very unpleasant*) to 100 (*very pleasant*). The order of the CS presentations and the questions was randomized across participants.

2.4. Physiological recordings and response definition

Postauricular reflex (PAR) and startle eyeblink reflex. The PAR was measured through electromyography (EMG) by pulling the left pinna forward and placing two 4-mm contact diameter Ag-AgCl electrodes filled with electrolyte gel on each side of the tendon of insertion for the PAR. One electrode was placed directly posterior to the tendon on the pinna surface, while the other electrode was placed over the postauricular muscle (Sollers & Hackley, 1997). The eyeblink reflex was measured through EMG recordings of the left orbicularis oculi muscle with two 4-mm contact diameter Ag-AgCl electrodes filled with electrolyte gel. Consistent with recent guidelines (Blumenthal et al., 2005), one electrode was placed below the lower left eyelid in line with the pupil in forward gaze and the second one 1-2 cm laterally. Two additional electrodes positioned on the top of the forehead were used as

recording reference and ground electrodes (see <http://www.biosemi.com/faq/cms&drl.htm>, for further information).

The EMG data was continuously recorded at 2048 Hz through a BIOSEMI Active-Two amplifier system (BioSemi Biomedical Instrumentation, Amsterdam, the Netherlands). The EMG analyses were carried out offline using Brain Vision Analyzer software (version 2.1; Brain Products GmbH, Gilching, Germany). Conventional bipolar montages were calculated from electrode pairs for the PAR and eyeblink reflex by subtracting the recorded activity of one electrode from the activity of the neighboring electrode. Prior to analysis, the PAR signal was bandpass (10-400 Hz) and notch filtered (50 Hz) before being rectified. The eyeblink reflex signal was bandpass (20-400 Hz) and notch filtered (50 Hz), rectified, and then low-pass filtered (40 Hz) (see Blumenthal et al., 2005). The filtered EMG signals were segmented into epochs from 100 ms prior to startle probe onset to 250 ms after probe onset. The 50 ms prior to startle probe onset were used as a baseline. Each segment was visually inspected and segments identified as containing excessive baseline shifts or blinks in progress were removed by hand from the analyses (4.16% of the trials for the PAR, and 4.16% of the trials for the eyeblink reflex).

Given its low signal-to-noise ratio as a microreflex, the PAR was scored after signal averaging of the rectified waveforms across trials within conditions (Aaron & Benning, 2016; Benning, 2011; Benning et al., 2004; Hackley et al., 1987, 2009; Hebert et al., 2015; Hess et al., 2007; Sollers & Hackley, 1997). The PAR magnitude was scored from the aggregate waveform as the baseline-to-peak amplitude for each condition. The peak was calculated as the maximum EMG activity occurring within a 5-35 ms time window after startle probe onset (Gable & Harmon-Jones, 2009; Sandt et al., 2009).

The startle eyeblink reflex was analyzed by means of a single-trial analysis, which corresponds to the most common method of analyzing eyeblink reflex data (Blumenthal et al.,

2005). Accordingly, the eyeblink reflex was scored for each trial as the baseline-to-peak amplitude of the maximum EMG activity occurring within 21-120 ms after startle probe onset (Blumenthal et al., 2005). The raw eyeblink scores were standardized within participants using T-scores. The eyeblink reflex magnitudes were calculated by averaging the T-scores for each condition.

Skin conductance response (SCR). SCR was measured with two 6-mm contact diameter Ag-AgCl electrodes filled with 0.5% NaCl electrolyte gel. The electrodes were attached to the distal phalanges of the second and third digits of the participants' nondominant hand. The SCR data was recorded at 2000 Hz through a BIOPAC MP150 system (Santa Barbara, California). The SCR analysis was performed offline with AcqKnowledge software (version 4.2; BIOPAC Systems Inc., Goleta, California). Before analysis, the SCR data was downsampled to 1000 Hz and low-pass filtered (1 Hz). SCR was scored for each trial as the peak-to-peak amplitude difference in skin conductance of the largest response occurring in the 0.5-4.5 s temporal window after CS onset. The minimal response criterion was 0.02 μ S. Responses below this criterion were scored as zero and remained in the analysis. SCRs were detected automatically with an AcqKnowledge routine and manually screened for artifacts and misdetections. The raw SCRs were square-root-transformed to reduce the distributions' positive skew. The square-root-transformed SCRs were then scaled according to each participant's maximal square-root-transformed SCR in order to take into account individual differences (Lykken & Venables, 1971). The habituation means included the first four presentations of each CS. The acquisition means comprised the nine presentations of each CS following the first pairing between the CS+ and the US. The extinction means were composed of the last eight presentations of each CS following the first US omission.

2.5 Statistical analyses

Paired *t*-tests were performed on the pleasantness and intensity ratings collected during the US selection procedure in order to ensure that the odor selected as the US was more pleasant and intense than odorless air. To assess whether there were differences in stimulus conditions in the conditioning phases, the PAR and the startle eyeblink reflex data were each analyzed with a one-way multivariate analysis of variance (MANOVA) with stimulus type (CS+ vs. CS- vs. ITI) as a within-participant factor and treating the habituation, acquisition, and extinction phases as multiple dependent variables. Separate one-way repeated measures ANOVAs with stimulus type (CS+ vs. CS- vs. ITI) as a within-participant factor were next conducted to investigate differences in stimulus conditions within each conditioning phase. Significant main effects were followed up with pairwise comparisons. To specifically test our a priori hypothesis, we performed a planned contrast comparing the PAR magnitude to the CS+ with the PAR magnitude to the CS- during acquisition. Likewise, we performed a planned contrast comparing the startle eyeblink reflex magnitude to the CS+ with the startle eyeblink magnitude to the CS- during the acquisition phase. Within each repeated measures ANOVA conducted, a stringent Bonferroni correction was applied on the pairwise comparisons' *p*-value to correct for multiple testing (i.e., $3 \times p$). SCR was analyzed separately for habituation, acquisition, and extinction with paired *t*-tests comparing the CS+ vs. the CS-. We additionally conducted an exploratory correlational analysis using Pearson's correlation coefficients to investigate whether (1) the PAR potentiation to the CS+ during acquisition and/or (2) the CS+/CS- differentiation as measured by the PAR were associated with participants' subjective hunger level. Finally, the CS-US contingency and the CS liking ratings were each analyzed with a paired *t*-test comparing the CS+ vs. the CS-.

An alpha level of .05 was adopted for all the statistical analyses performed. We provide the Huyhn-Feldt correction value (ϵ_{HF}) and the corrected *p*-value for the one-way

repeated measures ANOVAs. We moreover report either partial η^2 or Hedges' g_{av} as estimates of effect size (see Lakens, 2013) and their 90% or 95% confidence interval (CI), respectively.

3. Results

3.1 Olfactory US evaluation

The odor selected as the US was evaluated as more pleasant ($M = 83.84$, $SD = 13.53$) than odorless air ($M = 47.56$, $SD = 14.99$), $t(54) = 14.76$, $p < .001$, $g_{av} = 2.506$, 95% CI = [1.952, 3.122]. Likewise, the odor selected as the US was rated as more intense ($M = 70.19$, $SD = 16.59$) than odorless air ($M = 24.46$, $SD = 22.18$), $t(54) = 12.82$, $p < .001$, $g_{av} = 2.302$, 95% CI = [1.764, 2.896].

3.2 Postauricular reflex

The multivariate omnibus test revealed a statistically significant difference between the stimulus types in the conditioning phases, $F(6, 49) = 3.44$, $p = .006$, Wilk's $\Lambda = .703$, partial $\eta^2 = .297$, 90% CI = [.056, .380].¹ The one-way repeated measures ANOVA for the habituation phase revealed a statistically significant main effect of stimulus type, $F(2, 108) = 5.31$, $p = .007$, $\epsilon_{HF} = 0.98$, partial $\eta^2 = .090$, 90% CI = [.016, .173]. Follow-up comparisons showed that the PAR magnitude was greater during the ITI than to both the CS+, $t(54) = 3.01$, $p = .012$ (Bonferroni corrected), $g_{av} = 0.239$, 95% CI = [0.077, 0.406], and the CS-, $t(54) = 2.48$, $p = .048$ (Bonferroni corrected), $g_{av} = 0.224$, 95% CI = [0.042, 0.411] (see Figure 2A). These results replicate previous findings showing smaller PAR magnitudes during stimulus presentation than during ITIs (Benning, 2011; Benning et al., 2004), the PAR being generally inhibited by perceptual engagement with a stimulus (Benning, 2011; Hackley et al., 1987). Conversely, there was no statistical difference in PAR magnitude in response to the CS+ relative to the CS-, $t(54) = -0.11$, $p > .99$ (Bonferroni corrected), $g_{av} = -0.010$, 95% CI = [-0.184, 0.164] (see Figure 2A).

In the acquisition phase, a main effect of stimulus type was found, $F(2, 108) = 6.87, p = .003, \epsilon_{HF} = 0.80$, partial $\eta^2 = .113$, 90% CI = [.029, .201]. Congruent with our a priori hypothesis, the PAR magnitude was potentiated to the CS+ compared with the CS-, $t(54) = 2.97, p = .013$ (Bonferroni corrected), $g_{av} = 0.095$, 95% CI = [0.030, 0.161] (see Figure 2B). Further comparisons revealed that the PAR magnitude was greater during the ITI than to the CS-, $t(54) = 3.33, p = .005$ (Bonferroni corrected), $g_{av} = 0.166$, 95% CI = [0.063, 0.271], whereas there was no statistical difference in PAR magnitude during the ITI relative to the CS+, $t(54) = 1.47, p = .444$ (Bonferroni corrected), $g_{av} = 0.074$, 95% CI = [-0.027, 0.177] (see Figure 2B).

The one-way repeated measures ANOVA for extinction showed a statistically significant main effect of stimulus type, $F(2, 108) = 6.34, p = .004, \epsilon_{HF} = 0.89$, partial $\eta^2 = .105$, 90% CI = [.024, .192]. Follow-up comparisons revealed that the PAR magnitude was larger during the ITI than to the CS-, $t(54) = 3.35, p = .004$ (Bonferroni corrected), $g_{av} = 0.184$, 95% CI = [0.071, 0.301], and marginally larger than to the CS+, $t(54) = 2.28, p = .080$ (Bonferroni corrected), $g_{av} = 0.135$, 95% CI = [0.016, 0.257] (see Figure 2C). Importantly, the PAR magnitude was no longer potentiated in response to the CS+ compared with the CS-, $t(54) = 0.95, p > .99$ (Bonferroni corrected), $g_{av} = 0.043$, 95% CI = [-0.047, 0.134] (see Figure 2C).

INSERT FIGURE 2 ABOUT HERE

3.3 Startle eyeblink reflex

The one-way MANOVA yielded a statistically significant effect of stimulus type on the startle eyeblink reflex, $F(6, 49) = 5.91, p < .001$, Wilk's $\Lambda = .580$, partial $\eta^2 = .420$, 90% CI = [.174, .500].² During habituation, a statistically significant main effect of stimulus type was observed, $F(2, 108) = 6.33, p = .003, \epsilon_{HF} = 0.99$, partial $\eta^2 = .105$, 90% CI = [.024, .192]. Pairwise comparisons indicated that the startle eyeblink reflex magnitude was higher in

response to both the CS+, $t(54) = 2.81, p = .021$ (Bonferroni corrected), $g_{av} = 0.452$, 95% CI = [0.125, 0.788], and the CS-, $t(54) = 3.37, p = .004$ (Bonferroni corrected), $g_{av} = 0.633$, 95% CI = [0.247, 1.033], than during the ITI, reflecting that it was potentiated by the CSs (see Figure 3). However, there was no statistical difference in eyeblink reflex magnitude in response to the CS+ relative to the CS-, $t(54) = 0.86, p > .99$ (Bonferroni corrected), $g_{av} = 0.162$, 95% CI = [-0.213, 0.540] (see Figure 3).

Analysis of the acquisition phase showed a statistically significant main effect of stimulus type, $F(2, 108) = 8.94, p < .001, \epsilon_{HF} = 1$, partial $\eta^2 = .142$, 90% CI = [.047, .234]. The eyeblink reflex magnitude was however not attenuated in response to the CS+ compared with the CS-, $t(54) = 1.79, p = .237$ (Bonferroni corrected), $g_{av} = 0.304$, 95% CI = [-0.036, 0.650] (see Figure 3). Further comparisons revealed that the eyeblink reflex magnitude was greater to both the CS+, $t(54) = 2.47, p = .050$ (Bonferroni corrected), $g_{av} = 0.526$, 95% CI = [0.097, 0.966], and the CS-, $t(54) = 4.02, p < .001$ (Bonferroni corrected), $g_{av} = 0.842$, 95% CI = [0.404, 1.297], than during the ITI (see Figure 3).

In the extinction phase, a main effect of stimulus type was found, $F(2, 108) = 4.05, p = .020, \epsilon_{HF} = 1$, partial $\eta^2 = .070$, 90% CI = [.006, .147]. Follow-up comparisons showed that the CS- elicited a higher eyeblink reflex magnitude compared with the ITI, $t(54) = 2.64, p = .033$ (Bonferroni corrected), $g_{av} = 0.467$, 95% CI = [0.109, 0.834], whereas the eyeblink reflex magnitude to the CS+ was only marginally higher than during the ITI, $t(54) = 2.34, p = .068$ (Bonferroni corrected), $g_{av} = 0.442$, 95% CI = [0.062, 0.830] (see Figure 3). In addition, the eyeblink reflex magnitudes to the CS+ and to the CS- did not statistically differ, $t(54) = 0.04, p > .99$ (Bonferroni corrected), $g_{av} = 0.007$, 95% CI = [-0.342, 0.357] (see Figure 3).

INSERT FIGURE 3 ABOUT HERE

3.4 Skin conductance response

No preexistent difference was found in SCRs to the CS+ ($M = 0.07$, $SD = 0.11$) relative to the CS- ($M = 0.06$, $SD = 0.09$) during habituation, $t(50) = 0.71$, $p = .479$, $g_{av} = 0.097$, 95% CI = [-0.173, 0.369]. Similarly, SCRs to the CS+ ($M = 0.03$, $SD = 0.05$) were not larger than to the CS- ($M = 0.02$, $SD = 0.04$) during the acquisition phase, $t(50) = 0.88$, $p = .381$, $g_{av} = 0.113$, 95% CI = [-0.141, 0.369]. Analysis of the extinction phase likewise showed no statistical difference in SCRs to the CS+ ($M = 0.03$, $SD = 0.05$) compared with the CS- ($M = 0.03$, $SD = 0.05$), $t(50) = -0.52$, $p = .606$, $g_{av} = -0.073$, 95% CI = [-0.352, 0.206].³

3.5 Correlational analysis

The exploratory correlational analysis did not show that participants' subjective hunger level was associated either with the PAR magnitude to the CS+ during acquisition, $r(53) = .190$, $p = .165$, 95% CI [-0.079, .433], or with the CS+/CS- discrimination as measured by the PAR (i.e., PAR magnitude to the CS+ minus PAR magnitude to the CS-), $r(53) = .113$, $p = .412$, 95% CI [-0.157, .367].

3.6 Subjective ratings

Ratings of CS-US contingency revealed that the CS+ was rated as being more predictive of the olfactory US than the CS-, $t(54) = 4.78$, $p < .001$, $g_{av} = 0.944$, 95% CI = [0.522, 1.386] (see Figure 4A). In addition, ratings of CS liking showed that the CS+ was evaluated as more pleasant than the CS- after the extinction phase, $t(54) = 2.77$, $p = .008$, $g_{av} = 0.584$, 95% CI = [0.155, 1.024] (see Figure 4B).

INSERT FIGURE 4 ABOUT HERE

4. Discussion

In this study, we aimed to assess whether appetitive conditioning may be measured with the postauricular reflex in humans. We used a delay differential appetitive conditioning paradigm, in which a neutral stimulus (CS+) was systematically paired with a pleasant odor,

while another neutral stimulus (CS-) was not paired with any odor. We predicted that the postauricular reflex magnitude would be greater to the CS+ compared with the CS- during the acquisition phase. Taken together, our study provides initial evidence that the postauricular reflex can be used to index appetitive conditioning in humans.

First, subjective ratings show that we successfully induced appetitive conditioning in our participants. Overall, the CS+ was deemed more likely to be associated with the pleasant olfactory US than the CS-, indicating that participants were well aware of the contingencies between the CSs and the US. Moreover, the CS+ was evaluated as being more pleasant than the CS- after extinction. These evaluative effects highlight that appetitive conditioning had an impact on the CSs' subjective valence, and therefore demonstrate that the paradigm that we used was efficient in triggering appetitive conditioning.

Most importantly, our results indicate that the postauricular reflex constitutes a sensitive indicator of human appetitive conditioning. The postauricular reflex was indeed specifically potentiated in response to the CS+ compared with the CS- during acquisition, thereby reflecting appetitive learning at the psychophysiological level. This effect is consistent with prior findings that showed a greater postauricular reflex magnitude during presentation of pleasant/appetitive stimuli relative to neutral or unpleasant/aversive stimuli (Aaron & Benning, 2016; Benning, 2011; Benning et al., 2004; Dichter et al., 2010; Gable & Harmon-Jones, 2009; Hackley et al., 2009; Hess et al., 2007; Johnson et al., 2012; Sandt et al., 2009), and does not seem to have been related to participants' subjective hunger level. During the extinction phase, the postauricular reflex magnitude was no longer potentiated to the CS+ in comparison with the CS-, which suggests that its potentiation to the CS+ was conditioned to the pleasant odor delivery.

It is important to note that we were however not able to assess whether acquisition and extinction of the postauricular reflex potentiation to the CS+ occurred straight at the outset of

the acquisition and extinction phase, respectively, or more gradually. Because we analyzed the postauricular reflex data using signal averaging due to its low signal-to-noise ratio and did not probe every trial, a trial-by-trial analysis of the postauricular reflex modulation was neither possible nor warranted. Nonetheless, these results jointly suggest (1) that the postauricular reflex was sensitive to the contingency between the CS+ and the olfactory US, and (2) that the postauricular reflex magnitude modulation and the evaluative effects of appetitive conditioning potentially dissociated. This latter interpretation should nevertheless be considered with caution. As we did not measure ratings trial-by-trial, it is indeed possible that participants rated the conditioned stimuli according to their memories related to the acquisition phase, which might thus not reflect the actual pleasantness of the conditioned stimuli during or after extinction. However, since the CS+ was evaluated as more pleasant than the CS- after extinction, whereas the postauricular reflex potentiation to the CS+ extinguished when the pleasant odor was no longer delivered, our findings therefore do not provide evidence for the view that affective postauricular reflex modulation merely reflects the stimulus' subjective pleasantness *per se* (Gable & Harmon-Jones, 2009; Hebert et al., 2015). On the other hand, they rather suggest that the postauricular reflex indexes the predictive or current reward value of the stimulus at stake, which is likely to reflect the interplay of several components, without being limited to positive valence (see, e.g., Berridge & Robinson, 2003). In this respect, our study aligns with previous research suggesting that the postauricular reflex provides a valid psychophysiological indicator of motivational appetitive processes (Aaron & Benning, 2016; Benning, 2011; Benning et al., 2004; Hackley et al., 2009; Sandt et al., 2009).

As rewarding stimuli are typically arousing, it could be alternatively argued that the specific postauricular reflex potentiation to the CS+ relative to the CS- resulted from the CS+ being more arousing than the CS- during acquisition, and that the CS+ arousal value was

conversely no longer higher than the CS-'s during extinction. Although we cannot completely rule out this possibility, we do not think that the postauricular reflex was sensitive to the arousal dimension of the reward-related stimulus. Such an account of our data would indeed be inconsistent with previous findings in the postauricular reflex literature. Specifically, it has been reported that the stimulus' arousal level does not appear to modulate the postauricular reflex in response to pleasant or unpleasant stimuli (Gable & Harmon-Jones, 2009).

Appetitive-related stimuli have also been shown to evoke a greater postauricular reflex potentiation than non-appetitive pleasant stimuli, although both were reported as similarly arousing (Sandt et al., 2009). In addition, the fact that we observed no modulation of SCR, a prototypical measure of physiological arousal (e.g., Critchley, Elliott, Mathias, & Dolan, 2000), during the acquisition phase likewise does not align with the assumption that the postauricular reflex was modulated by arousal effects.

It should be noted that the greater postauricular reflex magnitude in response to the CS+ relative to the CS- could be conceptualized as a disinhibition of the postauricular reflex rather than a potentiation *per se*. This conceptualization seems to be consistent with the fact that the postauricular reflex magnitude was smaller in response to the conditioned stimuli than during the intertrial interval in the habituation phase, whereas the postauricular reflex magnitudes to the CS+ and during the intertrial interval were both greater than to the CS-, but did not statistically differ, in the acquisition phase. Putative neurophysiological processes responsible for this modulation pattern might involve a disinhibitory influence of appetitive stimuli within the postauricular reflex neural pathway that counteracts the reduced excitability of the neurons induced by perceptual engagement with a visual stimulus (see Hackley et al., 1987; Hackley, Ren, Underwood, & Valle-Inclán, 2017). The postauricular reflex neural circuitry is thought to comprise a disynaptic pathway from the cochlear root nucleus to the medial subdivision of the facial motor nucleus that, in turn, activates the postauricular muscle

(Hackley, 2015). Based on animal work on the pinna reflex (Li & Frost, 1996), the analog of the human postauricular reflex, it could be speculated that this disinhibitory influence is underlain by inputs from midbrain dopaminergic structures associated with reward processing (e.g., retrorubral nucleus; Waraczynski & Perkins, 2000) to the motoneurons of the facial nerve innervating the pinna (see Benning et al., 2004). However, further research is definitely needed to better understand the neurophysiological mechanisms of the postauricular reflex and elucidate whether its modulation to appetitive stimuli is best conceptualized as a potentiation or as a disinhibition.

With regard to the other psychophysiological measures collected, we found no evidence for startle attenuation in response to the CS+ relative to the CS- during the acquisition phase, and no effect of appetitive conditioning was observed on SCR. These results fail to replicate Andreatta and Pauli's (2015) study, which evidenced both startle attenuation and enhanced SCRs to the CS+ associated with the appetitive US relative to the CS-. However, this inconsistency might arise from several methodological disparities between this study and ours, including in particular the paradigm used (concurrent differential aversive and appetitive conditioning vs. differential appetitive conditioning only), as well as the conditioning procedure used during acquisition (compound conditioning vs. single-element conditioning). Another potential explanation relates to the use of a pleasant odor as appetitive US instead of food. Although both odors and food are primary rewards (Gottfried, 2011), odors constitute a generally less potent class of stimuli than food in humans. Consequently, appetitive olfactory conditioning might lead to smaller effects than appetitive food conditioning (see Rescorla & Wagner, 1972). In line with this proposition, Hermann et al. (2000) were unsuccessful in showing differential appetitive conditioning effects on startle eyeblink magnitude and SCR using a pleasant vanilla odor as US, which contrasts with Andreatta and Pauli's results using an appetitive food US.

Furthermore, other aspects can be equally advanced to account for the lack of statistically significant appetitive conditioning effects on the startle eyeblink reflex and SCR in our study: The startle response, as an aversive and defensive reflex (Lang et al., 1990), has been reported to be an unreliable indicator of appetitive processing in humans (Dichter et al., 2010; Dillon & LaBar, 2005; Jackson et al., 2000; for a review, see Grillon & Baas, 2003), while SCR, as a biomarker of autonomic arousal (Critchley et al., 2000), may be particularly sensitive to the US intensity, thereby possibly failing to consistently detect subtle changes caused by appetitive conditioning. Of note, the postauricular reflex has also been shown to be resistant to habituation (Hackley et al., 2017), which contrasts with the startle eyeblink reflex (e.g., Bradley, Lang, & Cuthbert, 1993; Grillon & Baas, 2003; Hackley et al., 2017; Rimpel, Geyer, & Hopf, 1982) and SCR (e.g., Bradley et al., 1993; Hare, Wood, Britain, & Shadman, 1971) that are both sensitive to habituation, and is thus less affected by repetitive stimulus presentations, as is the case in human conditioning paradigms. In sum, the fact that we observed differential appetitive conditioning at the psychophysiological level with the postauricular reflex suggests that it provides a sensitive psychophysiological measure of human appetitive conditioning, probably even more sensitive than both the startle eyeblink reflex and SCR.

Interestingly, whereas the postauricular reflex was inhibited by the presentation of the conditioned stimuli relative to the intertrial interval (see also Benning, 2011; Benning et al., 2004; Hackley et al., 1987), the opposite pattern of results was obtained for the startle eyeblink reflex, which was generally potentiated in response to the conditioned stimuli compared with the intertrial interval. This modulation pattern seems to align with previous reports in the human conditioning literature showing an overall greater startle eyeblink reflex magnitude to the CS- than during the intertrial interval (e.g., Andreatta & Pauli, 2015; Hamm, Greenwald, Bradley, & Lang, 1993). Given that startle modulation is affected by multiple

processes (Bradley, Codispoti, & Lang, 2006), it might possibly reflect the influence of attentional processes facilitating the enhancement of the acoustic eyeblink reflex during long lead intervals (e.g., when the interval between the stimulus onset and the startle probe is longer than three seconds), typically resulting in larger eyeblink reflex magnitude than during the intertrial interval (e.g., Lipp, Blumenthal, & Adam, 2001), or, alternatively, the impact of specific stimulus characteristics, such as perceptual complexity (see Stanley & Knight, 2004). However, such eyeblink reflex modulation pattern has not been consistently reported across human conditioning studies, some of which observing no enhanced startle eyeblink magnitude to the CS- relative to that during the intertrial interval for instance (see, e.g., Hamm & Vaitl, 1996; Lipp, Sheridan, & Siddle, 1994). This stresses that further investigation is required to better outline the determinants and the robustness of the eyeblink reflex modulation in response to (visual) conditioned stimuli vs. during the intertrial interval.

More generally, a caveat pertains to the number of trials included in each conditioning phase. In line with the current standards in the human conditioning literature (see, e.g., Lonsdorf et al., 2017), our study was specifically designed to assess changes between the different stimulus types used within each conditioning phase rather than between these phases. Therefore, we implemented a standard differential conditioning paradigm comprising fewer trials for each stimulus type in the habituation phase than in the acquisition and extinction phases, as is typically done in human conditioning paradigms (see, e.g., Andreatta & Pauli, 2015; Olsson, Ebert, Banaji, & Phelps, 2005). However, such differences in trial counts (and hence signal-to-noise ratios) may turn out to be somewhat problematic if one is interested in specifically testing whether the differences between the stimulus types are statistically different between the different conditioning phases (i.e., testing the interaction term). This issue especially holds for the postauricular reflex due to its relatively low signal-to-noise ratio. The postauricular reflex magnitude is likely to be considerably affected by the

number of aggregated trials when only few of them are eventually included per condition. In fact, the minimal amount of trials required for obtaining a reliable, stable measure of the postauricular reflex remains to be determined (but see Tooley, Carmel, Chapman, & Grimshaw, 2017, for a recent study suggesting that including at least 12 trials per condition seems to produce a robust estimate of the postauricular reflex magnitude). Those differences in trial numbers between phases (or conditions) may thus complicate the interpretation of the interaction effect, and even potentially produce statistically significant but spurious postauricular reflex magnitude differences. Consequently, future research aiming to specifically assess changes in psychophysiological responses to various stimulus types (e.g., CS+ vs. CS-) between the different conditioning phases should test and explicitly report such interaction term (or, alternatively, a planned contrast analysis, see Rosenthal & Rosnow, 1985), while ideally keeping the number of trials equal within each phase.

In conclusion, the present study suggests that the postauricular reflex arguably represents one of the most suitable psychophysiological indices for measuring appetitive conditioning in humans. In particular, the postauricular reflex sensitivity to appetitive contingencies indicates that this reflex is modulated by the stimulus' reward value, which supports its suitability as a measure of Pavlovian appetitive conditioning. These findings highlight that the postauricular reflex represents a promising psychophysiological indicator for studying Pavlovian reward learning, and more generally reward processing, in humans. Accordingly, future research should notably tackle in more detail whether the postauricular reflex provides a specific index for assessing – and potentially dissociating under particular circumstances – the distinct reward components of wanting, liking, and reward learning (see Berridge & Robinson, 2003; Pool, Sennwald, et al., 2016). Importantly, this research should however employ an appropriate concept operationalization of the reward components, and ideally take into account potential confounds (e.g., expected pleasantness; see Pool,

Sennwald, et al., 2016), along with the stimulus' affective relevance for the organism's concerns (see Pool, Brosch, Delplanque, & Sander, 2016; Pool, Sennwald, et al., 2016). In this perspective, the postauricular reflex constitutes a valuable tool for further shedding light on the basic mechanisms underlying appetitive conditioning and reward processing in humans, as well as their dysfunctions in specific disorders, such as depression, addiction, and food-related disorders.

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Footnotes

¹ Although the present study's aim was not to specifically assess changes between the stimulus types across the different conditioning phases, we nonetheless performed a two-way repeated measures ANOVA on the postauricular reflex data for the sake of completeness. This analysis revealed a statistically significant main effect of stimulus type, $F(2, 108) = 11.67, p < .001, \epsilon_{HF} = 0.90$, partial $\eta^2 = .178$, 90% CI = [.069, .279], and a marginal main effect of phase, $F(2, 108) = 2.97, p = .063, \epsilon_{HF} = 0.87$, partial $\eta^2 = .052$, 90% CI = [.000, .130], whereas the Stimulus type \times Phase interaction did not reach statistical significance, $F(4, 216) = 1.33, p = .266, \epsilon_{HF} = 0.81$, partial $\eta^2 = .024$, 90% CI = [.000, .057] (but see supplementary materials, for the outcome of more powerful planned contrasts testing specific patterns of results for the postauricular reflex).

² As for the postauricular reflex, we ran a two-way repeated measures ANOVA on the startle eyeblink reflex data for the sake of completeness. This analysis yielded statistically significant main effects of stimulus type, $F(2, 108) = 15.63, p < .001, \epsilon_{HF} = 1$, partial $\eta^2 = .225$, 90% CI = [.110, .322], and of phase, $F(2, 108) = 63.65, p < .001, \epsilon_{HF} = 0.83$, partial $\eta^2 = .541$, 90% CI = [.418, .621]. In contrast, the Stimulus type \times Phase interaction was not statistically significant, $F(4, 216) = 1.41, p = .239, \epsilon_{HF} = 0.83$, partial $\eta^2 = .025$, 90% CI = [.000, .059].

³ A two-way repeated measures ANOVA on the SCR data revealed a statistically significant main effect of phase, $F(2, 100) = 8.81, p = .002, \epsilon_{HF} = 0.70$, partial $\eta^2 = .150$, 90% CI = [.038, .270], reflecting a decrease in SCR magnitude from the habituation phase to the other conditioning phases. By contrast, the main effect of stimulus type was not statistically significant, $F(1, 50) = 0.41, p = .525, \epsilon_{HF} = 1$, partial $\eta^2 = .008$, 90% CI = [.000, .090], and no Stimulus type \times Phase interaction effect was observed, $F(2, 100) = 0.56, p = .511, \epsilon_{HF} = 0.70$, partial $\eta^2 = .011$, 90% CI = [.000, .073].

Table 1

Odors used in the unconditioned stimulus (US) selection procedure.

Odorant name	Odor family	Concentration (% in di- propylene glycol)	Mean liking (SD)	Mean intensity (SD)	Number of times selected as the US
Aladinate	Floral	50	32.95 (19.92)	63.49 (22.45)	0
Ariana	Detergent	20	64.69 (22.26)	66.96 (14.58)	10
Caramel	Sweet food	20	39.94 (25.01)	60.43 (19.27)	3
Chocolate	Sweet food	20	39.65 (26.38)	69.36 (20.88)	3
Galbex®	Floral	50	57.23 (21.69)	52.69 (22.04)	3
Geraniol	Floral	50	39.32 (22.17)	59.32 (22.81)	2
Green tea	Floral green	50	50.72 (15.16)	33.43 (24.65)	1
Lavender	Floral	20	46.14 (23.78)	61.74 (20.14)	1
Linalol	Floral	50	50.85 (20.89)	49.55 (24.40)	2
Magnolia grandiflora	Floral	50	53.29 (23.91)	60.91 (20.18)	4
Peach	Fruity	50	56.05 (21.35)	45.39 (21.40)	1
Pine	Woody	33	48.88 (19.88)	48.64 (24.09)	1
Pipol	Herbal	20	29.63 (20.79)	65.19 (24.76)	0
Speculaas	Sweet food	20	39.42 (22.85)	61.74 (19.24)	1
Strawberry	Fruity	20	58.88 (19.30)	60.27 (21.30)	4
Tiare	Floral	50	48.97 (22.02)	51.76 (24.26)	3
Tutti frutti	Fruity	20	64.69 (25.24)	62.48 (23.42)	16

Figure captions

Figure 1. Experimental design. (A) Geometric figures used as conditioned stimuli. (B) Conditioning phases. (C) Trial structure during the acquisition phase.

Figure 2. Grand-averaged postauricular reflex waveforms as a function of stimulus type (CS+ vs. CS- vs. ITI) across the (A) habituation, (B) acquisition, and (C) extinction phases.

Figure 3. Mean startle eyeblink reflex magnitudes as a function of stimulus type (CS+ vs. CS- vs. ITI) across the habituation, acquisition, and extinction phases. Error bars represent ± 1 standard error of the mean. *** $p < .001$, ** $p < .01$, * $p < .05$, ° $p < .10$ (Bonferroni corrected).

Figure 4. Mean (A) CS-US contingency ratings and (B) CS liking ratings after the appetitive conditioning procedure as a function of stimulus type (CS+ vs. CS-). Error bars represent ± 1 standard error of the mean. *** $p < .001$, ** $p < .01$.

Figure 1

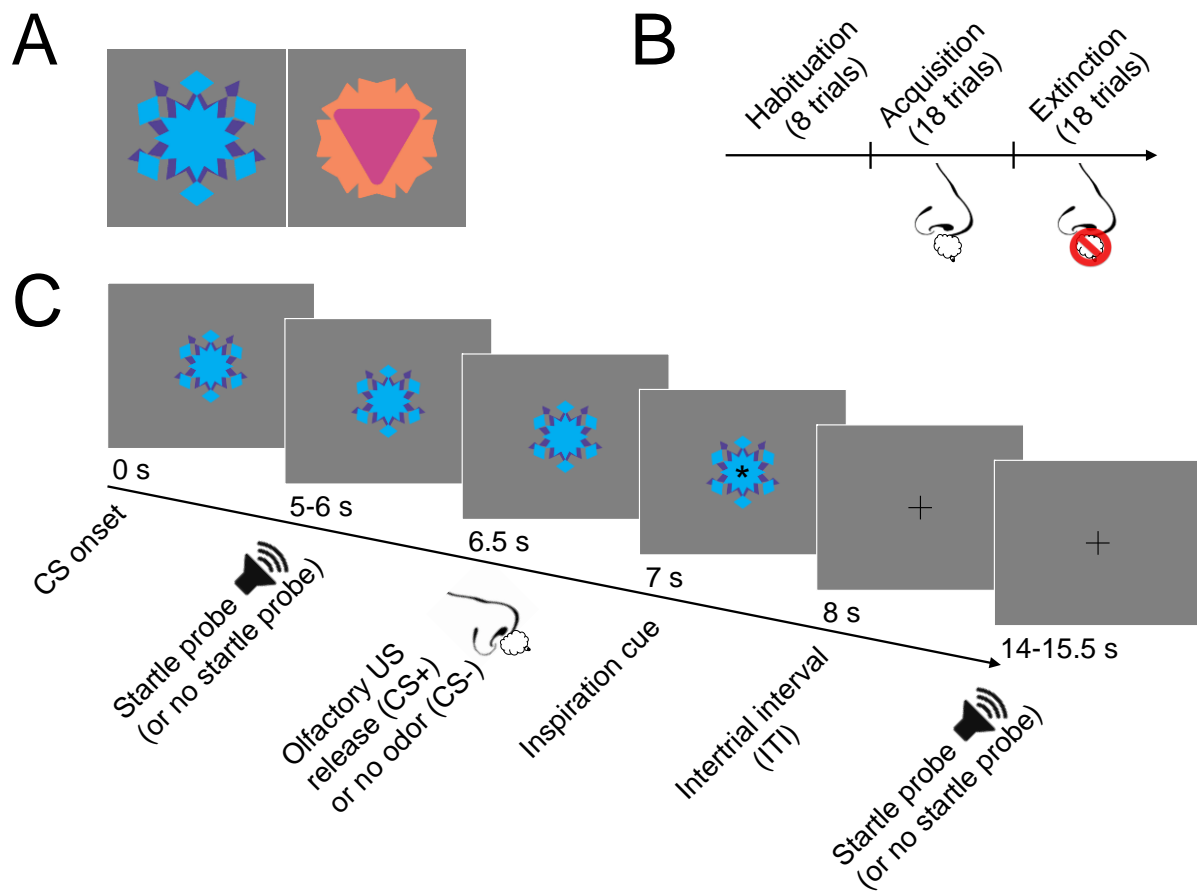


Figure 2

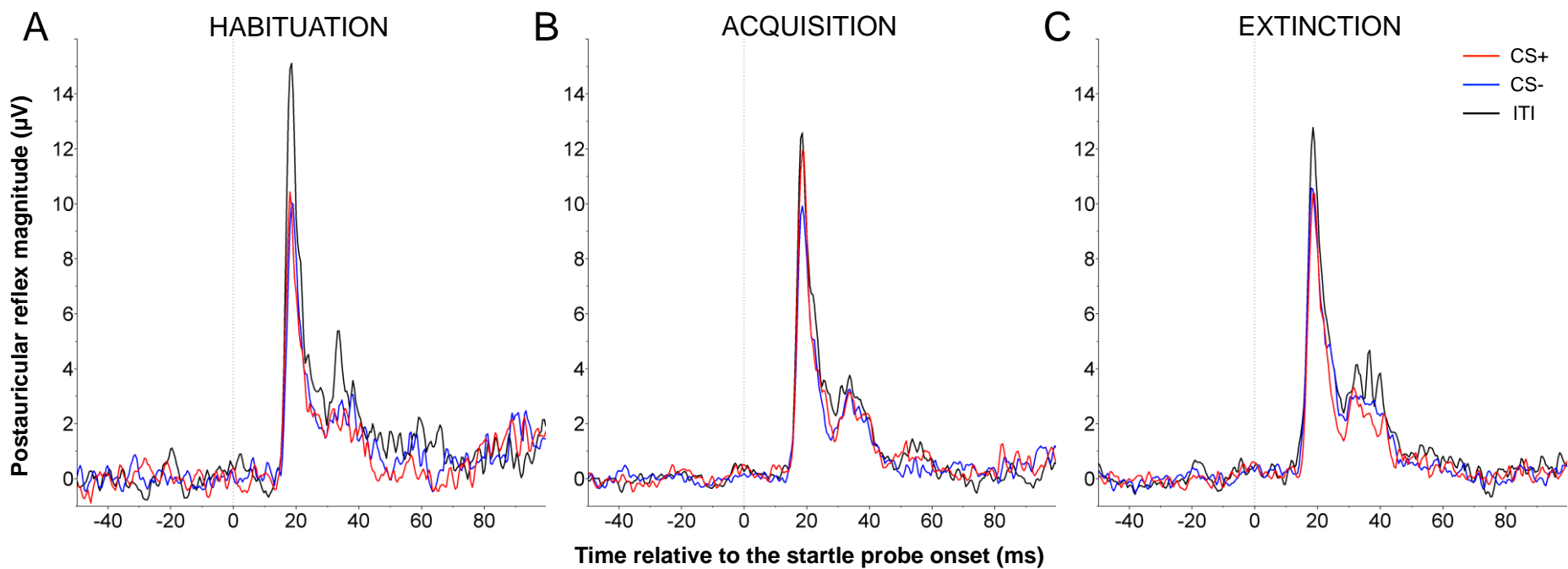


Figure 3

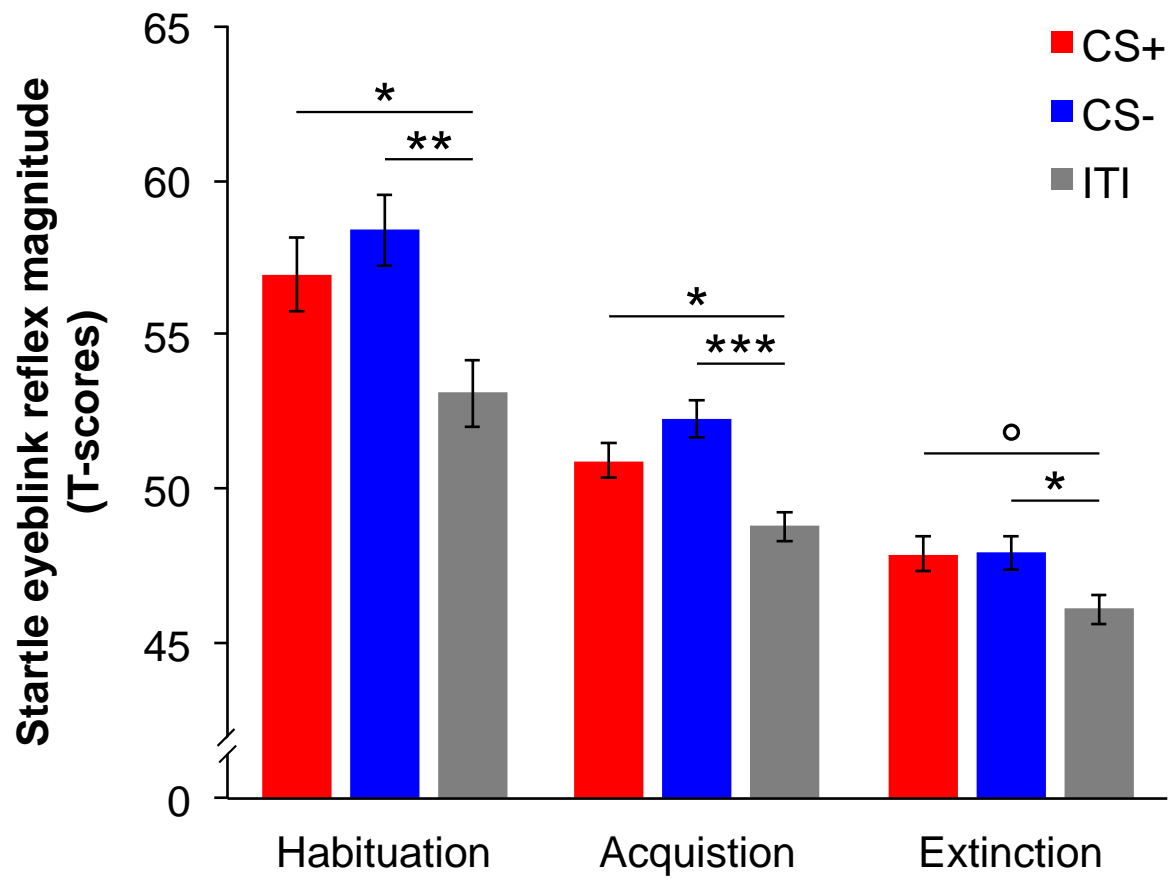


Figure 4

